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GENERAL PAPER

Development of a validated method for the simultaneous determination of amphetamine, methamphetamine and methylenedioxyamphetamines (MDA, MDMA, MDEA) in serum by GC-MS after derivatisation with perfluorooctanoyl chloride

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Abstract A rapid and simple method for the simultaneous detection and quantitation of amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) in human serum was developed and fully validated. Serum samples were extracted with cyclohexane, derivatised with perfluorooctanoyl chloride without prior evaporation of the solvent and analysed with gas chromatography-mass spectrometry (GC-MS) in the selected ion monitoring mode (SIM). For quantitation, deuterated analogues were used as the internal standards. The limit of detection (LOD) and lower limit of quantitation (LLOQ), bias and within-day and between-day precision were determined. LODs calculated as the average of the six calibration curves were below 5 ng/mL for all of the measured compounds; LLOQs obtained in the same manner were below 20 ng/mL, with the exception of MDA (24.1 ng/mL). The coefficients of variation were below 7% within series, 10% or less between series and the bias was below 8% for all compounds. The calibration curves were linear between the lower limits of quantitation and 800 ng/mL.

Keywords Amphetamines · Methylenedioxyamphetamines · GC-MS · Quantitation · PFOC-derivatisation after LLE

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Introduction

Amphetamines and their methylenedioxylated derivatives belong to a group of the most frequently abused drugs. The consumption of these substances as "party drugs," considered to be harmless by most young people, is alarming, especially if one considers their harmful effects on nerves, the liver, the kidneys and the brain. Fatal poisonings with and without the involvement of other toxicologically relevant substances have been reported [1–8]. Currently, there is an increasing number of publications dealing with drivers under the influence of methylenedioxyamphetamine-type drugs [9–13]. This development has affected street traffic law in Germany. According to §24a StVG, blood samples of supposedly drugged drivers are also routinely analysed for the presence of amphetamine, methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA). It is planned to extend this list in the near future to include methamphetamine as well. Furthermore, threshold values for prosecution are intended to be introduced at a level of 25 ng/mL for each of the amphetamines. In some forensic cases, the simultaneous determination of MDA, a metabolite of MDMA and MDEA, is also of great interest.

Numerous methods for the detection of amphetamines have been published in recent years (review of the years 1991 to 1997 [14]) using the following analytical techniques: high-pressure liquid chromatography (HPLC) [15–19], gas chromatography-mass spectrometry (GC-MS) [15, 20–25], liquid chromatography-mass spectrometry (LC-MS) [26, 27], capillary electrophoresis [28, 29], capillary electrophoresis-mass spectrometry [30] and high-field asymmetric waveform ion mobility spectrometry-mass spectrometry [31]. Most of these methods, however, involve evaporation of the extract prior to derivatisation.

Besides being time-consuming, this step can lead to a more or less pronounced loss of underivatised amphetamines [32].

The aim of this study was to develop a rapid, reliable, fully validated and low-priced method for the simultaneous detection of different amphetamines, especially for concentrations in the range of the proposed threshold value of 25 ng/mL.

In 1993, Gjerde et al. [33] published a GC-MS-method for the determination of amphetamine and methamphetamine in blood using derivatisation with perfluorooctanoyl chloride (pentadecafluorooctanoyl chloride, PFOC). This method, consisting of a simple liquid–liquid extraction with direct derivatisation of the extract with perfluorooctanoyl chloride without prior evaporation, was extended to allow the simultaneous analysis of the methylenedioxylated derivatives MDA, MDMA, and MDEA as well. In contrast to Gjerde et al. [33], who used only amphetamine-d_3 as the internal standard to quantitate amphetamine and methamphetamine in this procedure, the corresponding deuterated analogues of all target compounds were used. The perfluorooctanoyl-derivatives of methylenedioxyamphetamines and their deuterated analogues with high and characteristic ion masses have not been described yet. The method was fully validated following DIN 32645 and ISO 5725 (according to the guidelines of the Society of Toxicological and Forensic Chemistry, GTFCh) [32, 34, 35]. According to these guidelines, and in contrast to Gjerde et al. [33], three instead of two ion masses were recorded in single ion monitoring mode for each substance and the least intensive ions were used for the determination of limit of detection.

Materials and methods

Biological material

Serum used for preparing spiked samples to determine linearity, precision, accuracy, limit of detection (LOD) and lower limit of quantitation (LLOQ) was obtained from a volunteer who had given his formal consent.

Chemicals

Amphetamine sulphate, amphetamine-d_3 sulphate, methamphetamine hydrochloride, methylenedioxyamphetamine (MDA) hydrochloride, methylenedioxymethamphetamine (MDMA) hydrochloride and perfluorooctanoyl chloride (pentadecafluorooctanoyl chloride, PFOC) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Methanolic solutions of methylenedioxyethylamphetamine (MDEA), methamphetamine-d_8, MDA-d_5, MDMA-d_5 and MDEA-d_6 were purchased in concentrations of 100 µg/mL from Radian/Promochem (Wesel, Germany). All standards were obtained in a purity of 99% according to the certificate of analysis of the manufacturer. Cyclohexane p.a. and sodium hydroxide p.a. were purchased from Merck (Darmstadt, Germany).

Preparation of the internal standard mixture

Methanolic solutions of methamphetamine-d_8, MDA-d_5, MDMA-d_5 and MDEA-d_6 in concentrations of 100 µg/mL were purchased and used as stock solutions. A standard solution of amphetamine-d_3 was prepared by dissolving deuterated amphetamine sulfate in water to give a final amphetamine-d_3 concentration of 100 µg/mL. From these stock solutions, a mixture of deuterated standards with concentrations of 100 ng/mL (methamphetamine-d_8, MDA-d_5, MDMA-d_5) and 200 ng/mL (amphetamine-d_3, MDEA-d_6), respectively, was prepared.

Preparation of spiked serum samples

Aqueous stock solutions were prepared giving concentrations of 1 µg/mL and 100 ng/mL of each compound. These solutions were used to add 0, 5, 10, 15, 20 and 25 ng of each substance to 250 µL of blank serum, which corresponds to concentrations of 0, 20, 40, 60, 80 and 100 ng/mL serum. For the determination of the linearity range, concentrations of 200, 400, 600, 800 and 1,000 ng/mL of serum were also prepared using a stock solution containing 10 µg/mL of each compound.

Extraction procedure

To each spiked serum sample, 250 µL of the internal standard mixture, 500 µL of aqueous sodium hydroxide (5%, w/w) and 2 mL of cyclohexane were added. To prevent the formation of an emulsion, the mixture was gently vortex mixed or shaken by hand for about 30 s. After centrifugation (5 min, 4,000 rpm), about 1.5 mL of the organic layer were transferred to a glass tube. After adding 50 µL of PFOC, the glass tube was tightly closed with a Teflon-sealed screwcap. Derivatisation was performed at 70 °C for exactly 30 min. After evaporation of the solvent at 30 °C under a stream of compressed air, the residue was dissolved in 20 µL of dried and distilled ethyl acetate and transferred to a Teflon-sealed autosampler vial with a conical 300-µL insert for GC-MS analysis.

For the analysis of routine samples, 250 µL of serum were extracted in the same way.

Instrumentation

The GC-MS-system used was an Agilent 6890 gas chromatograph equipped with an Agilent 5973N mass selective detector (Böblingen, Germany), a Gerstel multipurpose sampler MPS2 (Mülheim an der Ruhr, Germany), a Chrompack CP7860 column (CP-Sil 5 CB, 30 m×250 µm i.d., 0.33-µm film thickness, Varian, Darmstadt, Germany) and HPCHEM software (G1034 C version C.03.00, Agilent Technologies, Waldbronn, Germany). Analyses were performed under the following conditions: injector temperature 250 °C, injection volume 1 µL in splitless mode, carrier gas helium, pressure-programmed flow rate 1.0 mL/min and MSD transferline 280 °C. The oven was temperature-programmed as follows: the initial temperature of 80 °C was held for 1 min, increased to 180 °C at a rate of 25 °C/min, then to 240 °C at a rate of 10 °C/min, then finally to 280 °C at a rate of 30 °C/min, and was held at this temperature for 4 min. The total run time was 16.33 min. The ionisation energy was 70 eV. The substances were measured in five groups in the selected ion monitoring mode (SIM) mode, as shown in Table 1, with a dwell time of 10 ms for each ion. The bold ions were used for quantitation.

Validation of the method

Statistical data were determined according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh) [32, 34, 35]. These prescribe that the LOD und LLOQ are calculated with a relative standard deviation ($1/k$) of 33.3% and confidence levels of 90% and 99%, respectively, using calibrator concentrations of 0, 20, 40, 60, 80 and 100 ng/mL. The validated method must fulfil the following demands for all analytes: correlation coefficient ≥0.98, LOD ≤15 ng/mL, LLOQ ≤25 ng/mL, within-day precision, between-day precision and bias ≤15%. Furthermore, the calibration should be linear up to a concentration of 1,000 ng/mL and recovery should be at least 50%.

All data were processed with the statistic software Valistat (version 1.0, Arvecon GmbH, Walldorf, Germany) [36], which is based on DIN 32645.

Table 1 Groups of selected ions monitored (m/z) and exemplary retention times of the pentadecafluorooctanoyl (PFO) derivatives

	Retention times (min)	Detected ions[a]
First group		
Amphetamine	6.35	118, **440**, 441
Amphetamine-d_3	6.34	121, **443**, 444
Second group		
Methamphetamine	7.00	118, 410, **454**
Methamphetamine-d_8	6.97	123, 413, **461**
Third group		
MDA	8.46	162, **440**, 575
MDA-d_5	8.44	167, **444**, 580
Fourth group		
MDMA	9.27	410, **454**, 589
MDMA-d_5	9.24	413, **458**, 594
Fifth group		
MDEA	9.57	162, 440, **468**
MDEA-d_6	9.54	165, 444, **474**

[a] The bold ions were used as the target ions for quantitation and for the determination of the lower limit of quantitation (LLOQ); for the determination of the limit of detection (LOD), the least intensive ions (underlined) were used

Selectivity

For the determination of method selectivity, ten different blank serum samples from different sources were analysed under the given GC-MS conditions to test for matrix interferences. In addition, three blank serum samples spiked with the internal standards were checked for the absence of analyte ions.

Linearity and homogeneity of variance

Six independently prepared calibration rows with a concentration range of 0–100 ng/mL consisting of spiked serum samples were analysed. Peak area ratios of the analytes to the internal standards were calculated as a function of the concentration. Additionally, in one series, concentrations up to 1,000 ng/ml were measured to determine the maximum linearity range. The replicates of each calibration level were checked for outliers via the Grubbs' test and then averaged. Using the mean values, calibration curves were checked for homoscedasticity (F test) and for linearity (Mandel test). Additionally, each calibration curve for each compound was tested for outliers and linearity, again using the F test and Mandel test. The confidence level used for each test was 99%.

Limit of detection and lower limit of quantitation

The limit of detection (LOD) and lower limit of quantitation (LLOQ) for concentrations up to 100 ng/mL were calculated according to DIN 32645 by interpreting the characteristics of the calibration curves. The least intensive ions (underlined in Table 1) were used for the determination of the LOD; the target ions (bolded in Table 1) were very specific and were used for the determination of the LLOQ. The LOD and LLOQ were calculated by using a relative standard deviation ($1/k$) of 33.3% and confidence levels of 90% (LOD) and 99% (LLOQ), respectively. The

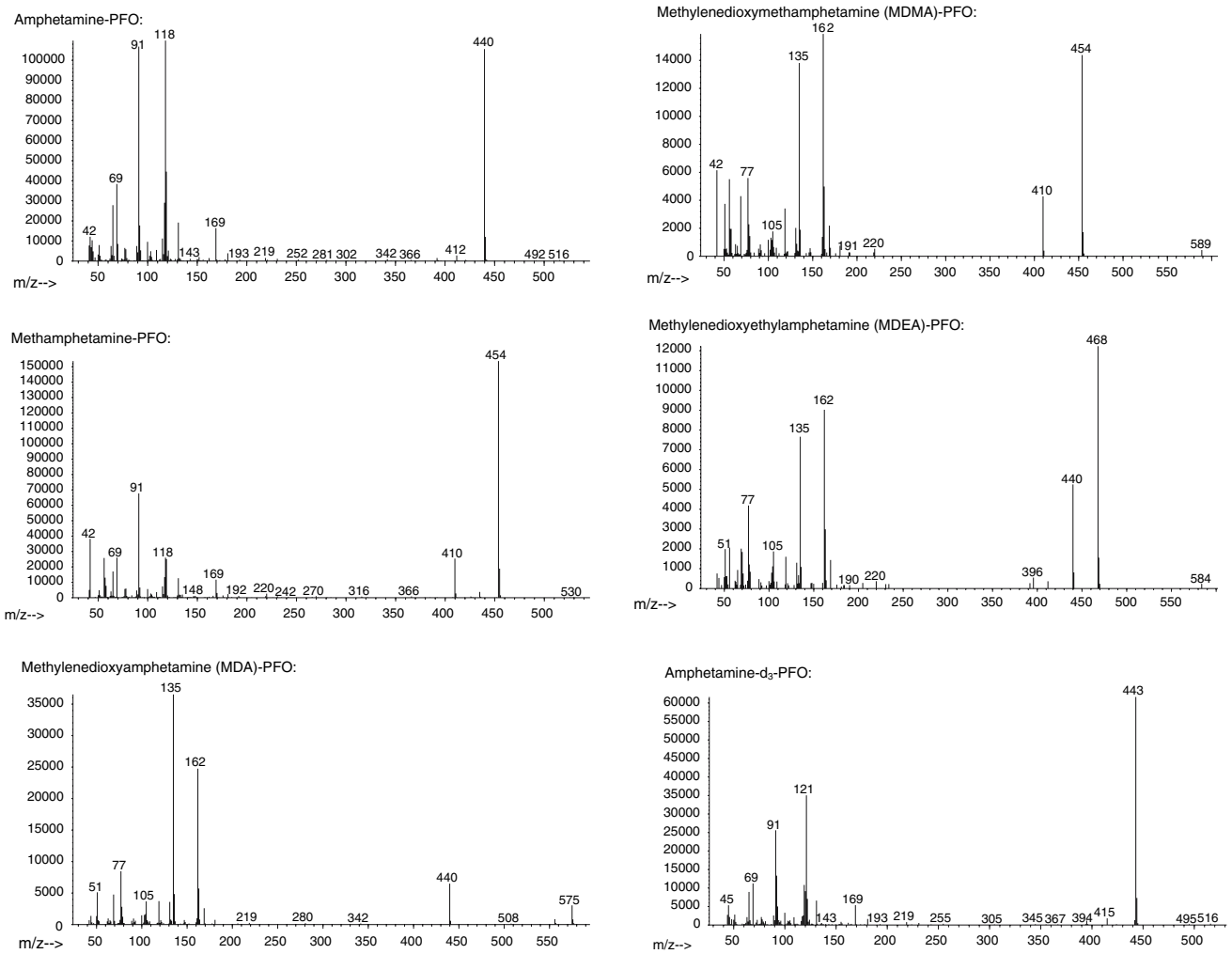

Fig. 1 Mass spectra of amphetamine-PFO, methamphetamine-PFO, MDA-PFO, MDMA-PFO, MDEA-PFO, amphetamine-d_3-PFO, methamphetamine-d_8-PFO, MDA-d_5-PFO, MDMA-d_5-PFO and MDEA-d_6-PFO

LOD and LLOQ shown in Table 2 were calculated as the average of the results of the six calibration sets.

Precision and accuracy

For the determination of within-day precision, between-day precision and accuracy (bias), spiked serum samples with concentrations of 20 ng/ml and 80 ng/ml, respectively, were analysed in duplicate on 8 days according to ISO 5725. The data were calculated by the analysis of variances (ANOVA) between groups test.

Recovery

Blank serum samples were spiked at concentrations of 20 ng/mL and 80 ng/mL for each compound and extracted as described above. Because the method requires only 250 μL of serum, this means that the samples contained 5 ng or 20 ng of each compound, respectively. Following centrifugation, as much as possible (usually between 1.5 mL and 1.8 mL) of the organic layer was removed to determine the maximum possible recoveries. After the addition of an internal standard mixture in cyclohexane, the samples were derivatised. Corresponding controls were prepared by adding the internal standard mixture and 5 ng or 20 ng of each compound to 1.5 mL of cyclohexane prior to derivatisation.

Results and discussion

Mass spectra and ion chromatograms

The mass spectra of amphetamine-PFO, methamphetamine-PFO, MDA-PFO, MDMA-PFO, MDEA-PFO and their corresponding deuterated internal standards measured under the given conditions are presented in Fig. 1.

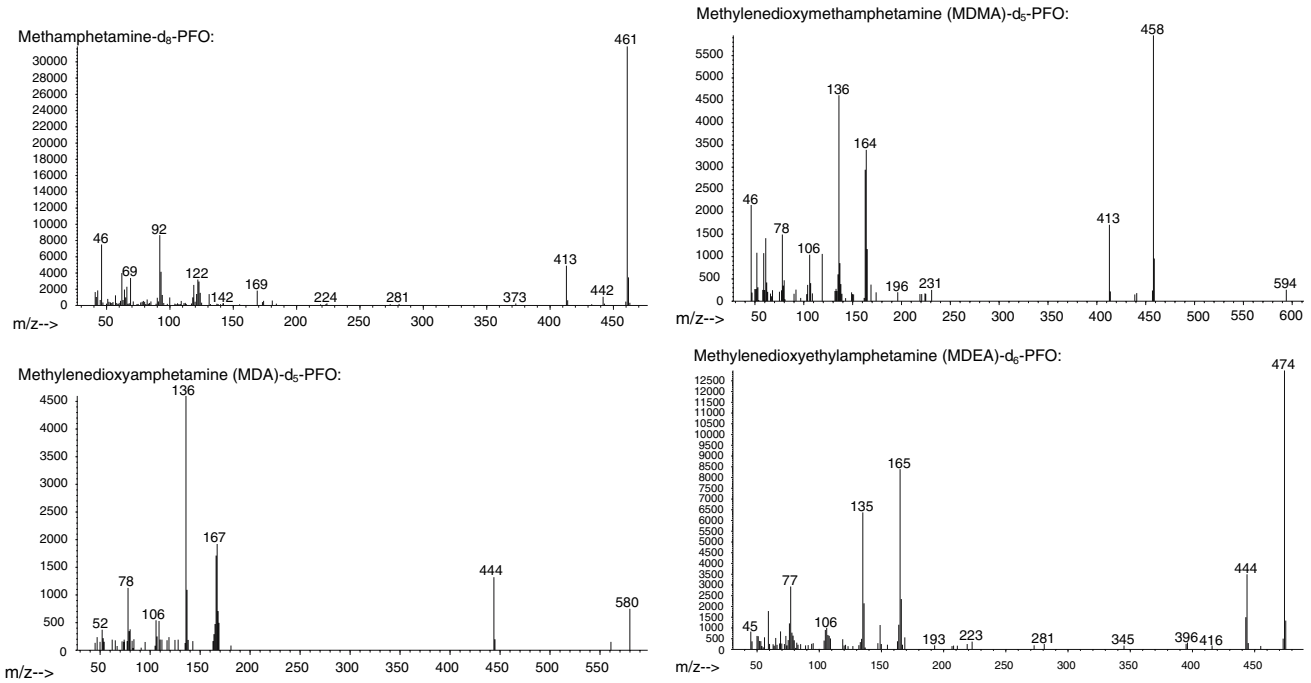

Fig. 1 continued

Table 2 Limits of detection (LOD) and lower limits of quantitation (LLOQ), accuracy (bias) and precision within and between series according to DIN 32645 and ISO 5725 under the given statistical parameters according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh)

Calibration range (0–100 ng/mL)	LOD (n=6) (mean)	LLOQ (n=6) (mean)	Within-day precision (n=8) (% RSD)		Between-day precision (n=8) (% RSD)		Bias (n=8) (%)	
	(ng/mL)	(ng/mL)	20 ng/mL	80 ng/mL	20 ng/mL	80 ng/mL	20 ng/mL	80 ng/mL
Amphetamine	3.5	11.6	2.2	3.1	5.7	4.3	2.1	1.1
Methamphetamine	2.4	16.0	5.0	1.6	7.1	3.5	6.2	1.3
MDA	2.5	24.1	6.3	1.6	10.0	4.5	7.6	0.8
MDMA	4.3	13.1	3.3	1.7	7.0	3.8	5.5	1.6
MDEA	1.4	13.2	4.8	2.3	7.6	3.0	0.1	0.1

The ion chromatograms of a serum sample of a drugged driver measured under the given conditions is shown in Fig. 2.

Validation data

All measurements were performed under routine analytical conditions. No interfering peaks from the serum matrix were observed. Peak purity and selectivity were ensured.

Validation data referring to the lower concentration range (up to 100 ng/mL) are presented in Tables 2 and 3. For the six calibration runs, a homoscedastic data set (constant variance over the whole range) was confirmed. The calibration curves were linear for all substances investigated. The LODs calculated as the average of the six calibration curves were below 5 ng/mL for all of the measured compounds. The LLOQs obtained in the same manner were below 20 ng/mL, with the exception of MDA. The values for precision and accuracy for extraction from the serum samples are expressed as relative standard deviation (RSD (%)). The coefficients of variation were below 7% within series and 10% or less between series; the bias was below 8% for all compounds (Table 2).

In one series, linearity was also determined for an extended calibration range using additional calibrators of 200, 400, 600, 800 and 1,000 ng/mL. Under these conditions, the calibration curves were linear for concentrations up to 800 ng/mL (Table 4); the 1,000 ng/mL calibrator was the first one to fail the linearity test.

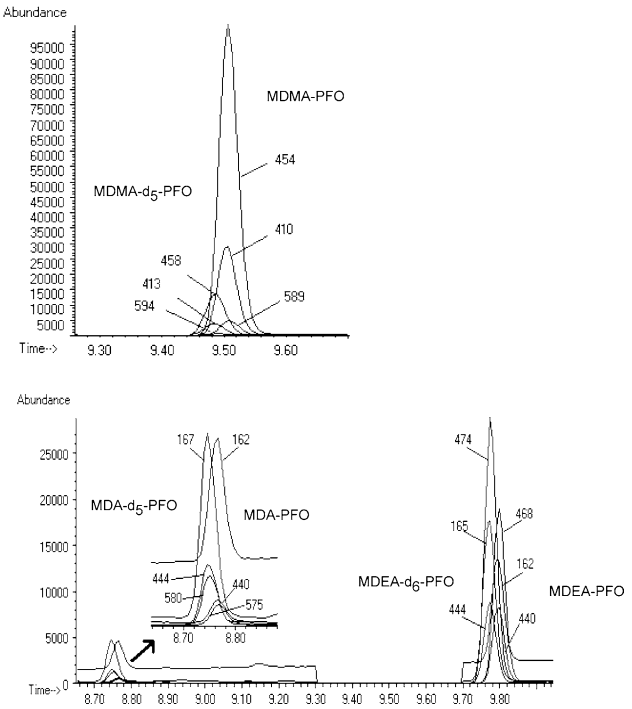

Fig. 2 Ion chromatograms of a serum sample of a drugged driver containing 577 ng/mL of MDMA, 38.3 ng/mL of MDA and 112 ng/mL of MDEA measured under the given conditions

The following mean recoveries were obtained at spiking concentrations of 20 ng/mL and 80 ng/mL, respectively (n=6): 88% and 80% for amphetamine (minima 78% and 65%), 92% and 79% for methamphetamine (minima 80% and 73%), 86% and 75% for MDA (minima 76% and 62%), 98% and 84% for MDMA (minima 89% and 72%) and 90% and 81% for MDEA (minima 77% and 69%). The lower recoveries at higher concentrations suggest that equilibrium has not quite been reached after an extraction time of 30 s. The obtained recoveries are, however, sufficient for the detection of even low concentrations of amphetamines. Therefore, to keep the method fast and to minimise the formation of emulsions, the extraction time was kept at 30 s.

A comparison of the target values proposed by the GTFCh and the observed values is given in Table 5. It could be shown that the validation fulfilled all of the mandatory regulations concerning the correlation coefficient, LOD, LLOQ, bias, within-day and between-day precision for each analyte. The recommendation concerning recovery was also completely met, whereas the calibration curves turned out to be linear only up to 800 ng/mL.

Conclusion and outlook

The reliability of analytical findings is a matter of great importance in forensic and clinical toxicology, as the results may have wide legal consequences or lead to the wrong treatment of a patient. So, at the very least, routine analytical methods have to be validated, preferably using a procedure which focusses on those parameters which are of special importance for toxicologists [37]. The guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh) meet these requirements.

By means of the rapid and simple method presented above, amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) can be quantitated simultaneously in serum, even at low concentration levels. Using the corresponding deuterated analogues of the target compounds as internal standards resulted in lower values for the limit of detection (LOD), the lower limit of quantitation (LLOQ) and precision data for amphetamine and methamphetamine in comparison to the published method of Gjerde et al. [33]. The validation data show that the method yields very reliable and repro-

Table 3 Linearity parameters for a calibration range of 0–100 ng/mL (n=6). The results are given for the target ion (standard font) and the least intensive ion (in italics)

	Amphetamine	Methamphetamine	MDA	MDMA	MDEA
Correlation coefficients (ranges)	0.9996–0.9999	0.9991–0.9998	0.9989–0.9995	0.9996–0.9999	0.9996–0.9998
	0.9984–0.9995	*0.9988–0.9998*	*0.9993–0.9997*	*0.9967–0.9996*	*0.9998–0.9999*
Standard error of the regression (ranges)	0.0015–0.0052	0.0075–0.0215	0.0132–0.0210	0.0050–0.0119	0.0038–0.0064
	0.0066–0.0118	*0.0099–0.0266*	*0.0131–0.0199*	*0.0144–0.0447*	*0.0041–0.0076*
Slope (mL/ng) (mean±SD[a])	0.0050 ± 0.0001	0.0123 ± 0.0005	0.0109 ± 0.0012	0.0109 ± 0.0004	0.0060 ± 0.0002
	0.0049 ± 0.0003	*0.0129 ± 0.0006*	*0.0129 ± 0.0006*	*0.0131 ± 0.0011*	*0.0087 ± 0.0003*
y intercept (mean±SD[a])	–0.0005 ± 0.0081	–0.0003 ± 0.0292	0.0049 ± 0.0368	–0.0020 ± 0.0203	0.0013 ± 0.0112
	0.0019 ± 0.0203	*0.0002 ± 0.0363*	*–0.0043 ± 0.0380*	*0.0019 ± 0.0700*	*0.0000 ± 0.0153*
Linear range (ng/mL)	3.5–100	2.4–100	2.5–100	4.3–100	1.4–100

[a] 95% confidence interval

Table 4 Linearity parameters for a calibration range of 0–800 ng/mL. Results are given for the target ion (standard font) and the least intensive ion (in italics)

	Amphetamine	Methamphetamine	MDA	MDMA	MDEA
Correlation coefficients	0.9998	0.9983	0.9985	0.9996	0.9996
	0.9997	*0.9993*	*0.9998*	*0.9997*	*0.9997*
Standard error of the regression	0.0288	0.3101	0.2187	0.0885	0.0655
	0.0386	*0.2134*	*0.0877*	*0.0963*	*0.0891*
Slope (mL/ng) (±SD[a])	0.0050 ± 0.0001	0.0144 ± 0.0006	0.0111 ± 0.0004	0.0116 ± 0.0002	0.0066 ± 0.0001
	0.0051 ± 0.0001	*0.0152 ± 0.0004*	*0.0134 ± 0.0002*	*0.0135 ± 0.0003*	*0.0094 ± 0.0002*
y intercept (±SD[a])	–0.0179 ± 0.0279	–0.2206 ± 0.2840	–0.0807 ± 0.2004	–0.0697 ± 0.0857	–0.0592 ± 0.0600
	–0.0219 ± 0.0374	*–0.1951 ± 0.1955*	*–0.0784 ± 0.0803*	*–0.0627 ± 0.0933*	*–0.0649 ± 0.0816*
Linear range (ng/mL)	3.5–800	2.4–800	2.5–800	4.3–800	1.4–800

[a] 95% confidence interval

Table 5 Comparison of the target values and the observed values

Parameter	Target value	Observed values
Correlation coefficient	≥0.98	>0.99
Limit of detection (LOD)	≤15 ng/mL	≤4.3 ng/mL
Lower limit of quantitation (LLOQ)	≤25 ng/mL	≤20.0 ng/mL (MDA 24.1 ng/mL)
Within-day precision	≤15%	≤6.3%
Between-day precision	≤15%	≤10.0%
Bias	≤15%	≤7.6%
Recovery	≥50%[a]	≥75%
Linearity	up to 1,000 ng/mL[a]	up to 800 ng/mL

[a] Recommendations

ducible results. The in-situ derivatisation without prior evaporation saves time and prevents losses of the highly volatile underivatised amphetamines. The high recoveries allow for an extremely short extraction time of about 30 s. Large series of samples can be measured in a short time, with low costs and only a small amount of serum sample is needed.

During the past few years, the method has been effectively employed in the routine analysis of hundreds of serum samples from drivers suspected to be under the influence of amphetamine or its derivatives and also for proficiency tests. Furthermore, the method has been successfully utilised for the analysis of hairs after alkaline dissolution and of urine samples. These results suggest that the presented method might also be used for the analysis of other biological materials in forensic and clinical cases.

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